Express Mail Label No. EL 405 597 762 US

2-2-00

A

JC377 U.S. PTO 01/31/00

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No. 0769.00136
Total Pages in this Submission

TO THE ASSISTANT COMMISSIONER FOR PATENTS
Box Patent Application
Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for an invention entitled:

ASSAYS FOR TSH RECEPTOR AUTOANTIBODIES

JC690 U.S. PTO 09/494751 01/31/00

and invented by:

Jane Sanders; Bernard Rees Smith; Jadwiga Furmaniak

If a CONTINUATION APPLICATION, *check appropriate box and supply the requisite information:*

☐ **Continuation** ☐ **Divisional** ☒ **Continuation-in-part (CIP)** of prior application No.: **PCT/GB99/01774**

Which is a:

☐ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: _____

Which is a:

☐ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: _____

Enclosed are:

Application Elements

1. ☒ Filing fee as calculated and transmitted as described below
2. ☒ Specification having **25** pages and including the following:
   a. ☒ Descriptive Title of the Invention
   b. ☒ Cross References to Related Applications *(if applicable)*
   c. ☐ Statement Regarding Federally-sponsored Research/Development *(if applicable)*
   d. ☐ Reference to Microfiche Appendix *(if applicable)*
   e. ☒ Background of the Invention
   f. ☒ Brief Summary of the Invention
   g. ☒ Brief Description of the Drawings *(if drawings filed)*
   h. ☒ Detailed Description
   i. ☒ Claim(s) as Classified Below
   j. ☒ Abstract of the Disclosure

EL405597762US

1-31-00

with the United States Postal Service Post Office To Addressee on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Constance McLean

(Signature of person mailing paper or fee)

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No. 0769.00136
Total Pages in this Submission

Application Elements (Continued)

3. ☒ Drawing(s) *(when necessary as prescribed by 35 USC 113)*

   a. ☐ Formal b. ☒ Informal Number of Sheets 3

4. ☒ Oath or Declaration

   a. ☒ Newly executed *(original or copy)* ☐ Unexecuted

   b. ☐ Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional application only)*

   c. ☒ With Power of Attorney ☐ Without Power of Attorney

   d. ☐ *DELETION OF INVENTOR(S)*
   Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. 1.63(d)(2) and 1.33(b).

5. ☐ Incorporation By Reference *(usable if Box 4b is checked)*
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Computer Program in Microfiche

7. ☐ Genetic Sequence Submission *(if applicable, all must be included)*

   a. ☐ Paper Copy

   b. ☐ Computer Readable Copy

   c. ☐ Statement Verifying Identical Paper and Computer Readable Copy

Accompanying Application Parts

8. ☒ Assignment Papers *(cover sheet & documents)*

9. ☐ 37 CFR 3.73(b) Statement *(when there is an assignee)*

10. ☐ English Translation Document *(if applicable)*

11. ☐ Information Disclosure Statement/PTO-1449 ☐ Copies of IDS Citations

12. ☐ Preliminary Amendment

13. ☒ Acknowledgment postcard

14. ☒ Certificate of Mailing

    ☐ First Class ☒ Express Mail *(Specify Label No.):* EL 405 597 762 US

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
0769.00136

Total Pages in this Submission

Accompanying Application Parts (Continued)

15. ☐ Certified Copy of Priority Document(s) *(if foreign priority is claimed)*

16. ☒ Small Entity Statement(s) - Specify Number of Statements Submitted: **2**

17. ☐ Additional Enclosures *(please identify below):*

Fee Calculation and Transmittal

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra		Rate	Fee
Total Claims	62	- 20 =	42	x	$9.00	$378.00
Indep. Claims	2	- 3 =	0	x	$39.00	$0.00
Multiple Dependent Claims (check if applicable) ☐						$0.00
					BASIC FEE	$345.00
OTHER FEE *(specify purpose)*	**Assignment Recordation Fee**					$40.00
					TOTAL FILING FEE	$763.00

☒ A check in the amount of **$763.00** to cover the filing fee is enclosed.

☒ The Commissioner is hereby authorized to charge and credit Deposit Account No. **11-1449** as described below. A duplicate copy of this sheet is enclosed.

- ☐ Charge the amount of as filing fee.
- ☒ Credit any overpayment.
- ☒ Charge any additional filing fees required under 37 C.F.R. 1.16 and 1.17.
- ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: **January 31, 2000**

Signature

Kenneth I. Kohn, Reg. No.30,955
KOHN & ASSOCIATES
30500 Northwestern Highway, Suite 410
Farmington Hills, Michigan 48334
(248) 539-5050
FAX: (248) 539-5055

cc:

Assays for TSH Receptor Autoantibodies

CROSS REFERENCE TO RELATED APPLICATION

The present application is a continuation in part of International Patent Application PCT/GB99/01774 filed on 4th June 1999 by the same applicants as the present invention.

BACKGROUND OF THE INVENTION

The present invention is concerned with assays (kits and analytical methods) for detecting or monitoring TSH Receptor Autoantibodies.

The hyperthyroidism which is associated with Graves' disease is known to be due to autoantibodies to the thyroidal receptor for thyroid stimulating hormone (TSH). The autoantibodies bind to the receptor and mimic the actions of the natural ligand (TSH), thereby causing the gland to produce high levels of thyroid hormones (as described in Endocrine Reviews 1988, Vol 9, No. 1, pages 106 to 117).

The detection or monitoring of TSH receptor autoantibodies (TRAb) is important in the diagnosis and management of Graves' disease and currently two types of assay are used, namely:

(a) competitive binding assays which measure the ability of TRAb to inhibit the binding of ^{125}I-labelled TSH to preparations of TSH receptor; and

(b) bioassays which measure the ability of TRAb to stimulate thyroid cells (or other cells transfected with the TSH receptor gene) in culture.

Currently, competitive binding assays (type (a) above) are more widely used, because bioassays of the type mentioned in (b) above are expensive, time-consuming, require highly skilled staff and are unsuitable for wide routine use. In current competitive binding assays, test serum samples (50μl) are generally incubated with detergent solubilized porcine TSH receptor (50 μl). TRAb present in the test sera bind to the TSH receptor during this incubation. ^{125}I-labelled TSH is then added and the incubation continued. During this second incubation, the labelled TSH binds to TSH receptors not already occupied by TRAb. Finally, ^{125}I-labelled TSH bound to the receptor is separated from free labelled TSH by addition of

polyethylene glycol (PEG), which precipitates the receptor bound TSH but not the free TSH. The radioactivity in the precipitates (separated by centrifugation) is then counted. In the assay, TRAb in test samples inhibits the binding of labelled TSH to the TSH receptor and this results in a lowering of the radioactivity in the precipitates. Assay results can be expressed as an index of inhibition of labelled TSH binding or by use of a set of assay calibrators.

The main limitations of this conventional assay are as follows:

(a) The assay measures competition between labelled TSH and the TSH receptor and may not detect TRAb which bind well to the receptor but in such a way as not to inhibit TSH binding strongly.

(b) The assay uses polyethylene glycol to separate receptor bound and free labelled TSH. This results in co-precipitation of all the serum immunoglobulins and the formation of a relatively large pellet. Although the pellet can be counted for radioactivity, it is not a suitable preparation to detect TSH (or other proteins or peptides) labelled with non-radioactive substances such as enzymes or chemiluminescent materials. This is because the serum components in the pellet interfere with such processes as light emission. In addition, the use of PEG precipitation necessitates the use of centrifugation and this is a time-consuming and cumbersome procedure unsuitable for automation.

Published patent specification EP0719858A describes an assay method for TSH receptor autoantibodies, where TSH receptor is bound to a solid phase either directly or via anti-TSH receptor antibody. A method for determination of TSH receptor autoantibodies present in patient serum is also described in published patent specification WO95/06258.

OBJECTS OF THE INVENTION

It is an object of the present invention to provide an improved assay for TRAb of the competitive binding type.

It is a further object of the present invention to provide an improved assay kit of the competitive binding type for monitoring TRAb present in a sample of body fluid.

It is a further object of the present invention to provide an improved TRAb assay of the direct binding type, in which a direct interaction between the receptor and TRAb is used.

It is a further object of the present invention to provide an improved assay kit of the direct binding type for monitoring TRAb present in a sample of body fluid.

SUMMARY OF THE INVENTION

The present invention provides a method of monitoring autoantibodies to thyroid stimulating hormone (TSH) receptor in a sample of body fluid, comprising, in the following order, the steps of:

(a) providing (i) porcine TSH receptor or a fragment thereof immobilized to a solid phase, or (ii) TSH receptor which is complexed to a labelled antibody;

(b) incubating the TSH receptor with a sample of body fluid;

(c) reacting the incubated sample of body fluid containing the TSH receptor with at least one binding agent which is capable of binding to the TSH receptor in competitive reaction with TSH receptor autoantibodies (TRAb), or when the TSH receptor is (ii), reacting the sample of body fluid, during or after step (b), with at least one binding agent which can bind to TRAb in such a way as not substantially to interfere with binding of the TRAb to the TSH receptor; and

(d) qualitatively or quantitatively detecting bound TRAb in the reacted incubated sample of body fluid.

The sample of body fluid typically comprises blood, plasma or serum.

The invention comprises the use of antibodies in order to label or immobilize a TSH receptor, the immobilized or labelled TSH receptor being such that it retains its ability to bind TSH and/or TRAb.

The present invention preferably concerns the use of a monoclonal (or polyclonal) antibody to the TSH receptor, which is bound strongly to the receptor at a site distinct from the part of the receptor which binds TSH and TRAb. The antibody can bind to the receptor strongly at the same time as TSH or TRAb and can be used to alleviate many of the limitations of the current TRAb assay method.

In a first embodiment of the invention, the antibody can be immobilized on a solid phase (such as a plastic tube or plastic plate, or magnetic or non-magnetic particles) using standard procedures. This solid phase can then be used instead of PEG to separate labelled TSH bound to the TSH receptor from free labelled TSH. The TSH (or similar ligand) can be labelled with isotopic or non-isotopic labels.

There are several applications of this first embodiment, of which the following are schematic examples:

(i) TSH receptor + labelled TSH ⟶ TSH receptor - labelled TSH

↑ Binding inhibited by TRAb

↓ separate from unbound labelled TSH by addition of immobilized receptor antibody (□-Ab)

↓

□-Ab-TSH receptor-labelled TSH

(ii) □-Ab-TSH receptor + labelled TSH ⟶ □-Ab-TSH receptor-labelled TSH

Form this complex first ↑ binding inhibited by TRAb

(iii) In (i) and (ii) above, the receptor Ab can be bound **indirectly** to the solid phase. For example, the Ab can be biotinylated and reacted with the solid phase which contains avidin or streptavidin:

(a) Bi-Ab-TSH receptor + labelled TSH ⟶ Bi-Ab-TSH receptor-labelled TSH complex

↑ binding inhibited by TRAb

↓ separate from unbound labelled TSH by addition of immobilized avidin or streptavidin (□-Avidin)

↓

□-Avidin-Bi-Ab-TSH receptor-labelled TSH

(b) □-Avidin-Bi-Ab-TSH receptor + labelled TSH ⟶ □-Avidin-Bi-Ab

Form this complex first

↑ binding inhibited by TRAb

| TSH receptor labelled TSH

In a second embodiment of the invention, the antibody can be labelled directly with an isotopic label such as ^{125}I, or with a non-isotopic label such as an enzyme, dye, or chemiluminescent compound. Alternatively, the antibody may be labelled indirectly using, for example, the avidin-biotin system. The labelled antibody can then be used to label the TSH receptor itself and this complex of receptor and antibody can then be used to detect and/or monitor TRAb (either qualitatively or quantitatively).

Examples of this approach are described schematically as follows:

(i) Labelled Ab - TSH receptor + TRAb ⟶ labelled Ab-TSH receptor

Form this complex first

|

TRAb

↓

Add anti-human IgG reagent such as solid phase protein A (□-protein A)

↓

Labelled Ab-TSH receptor

|

□ - protein A - TRAb

In this system, increasing amounts of TRAb in a test sample will result in increasing amounts of labelled Ab in the final complex.

(ii) This example depends on the coupling of TSH (or similar ligand) directly or indirectly to a solid phase (□-TSH) receptor labelled with the antibody (which is itself labelled with an isotopic or non-isotopic label), which can bind to this immobilized TSH (or similar ligand). This binding will be inhibited by TRAb, as illustrated below:-

□ - TSH + TSH receptor - labelled Ab ----> □ - TSH - TSH receptor

immobilized TSH ↑ |

binding inhibited by TRAb — labelled Ab

The schemes described above with reference to the first and second embodiments of the invention illustrate certain advantages of the present invention. The immobilized antibody may be used to label or immobilize the TSH receptor in such a way that the receptor retains its ability to bind TSH and/or TRAb. Alternatively, the immobilized antibody may be used for the monitoring of TRAb (or other ligands which interact with the

TSH receptor) in patient sera, or for purification of the TSH receptor.

In particular, the ability to label the TSH receptor using the antibody permits monitoring of the direct interaction of TRAb with the TSH receptor as in the second embodiment of the invention described above. Furthermore, the present invention allows the immobilization of the receptor before, during, or after interaction with TSH and TRAb. This ability to immobilize the receptor can be used to create new TRAb assays which do not depend on PEG and/or isotopic labels. Such assays may be suitable for automation and immunochromatographic systems.

The TSH receptor (TSHR) is present in very low numbers on the surface of thyrocytes (about 10^3 receptors per cell) which has made the receptor very difficult to purify from native sources (as described in "Baillière's Clinical Endocrinology and Metabolism", 1997, Vol II, pages 451 to 474 - Sanders).

In contrast, recombinant TSHR can be expressed in mammalian cells (for example, in Chinese hamster ovary (CHO) cells) at much higher levels of about 10^5-10^6 receptors per cell (Sanders). In addition, recombinant TSHR preparations produced in non-thyroid cells are not contaminated with other thyroid autoantigens such as thyroglobulin or thyroid peroxidase (Springer Seminars in Immunopathology, 1993, pages 309-318 - Furmaniak).

Recombinant TSHR preparations produced in mammalian cells are the only ones which show TSH and TRAb binding characteristics similar to that of the native receptors. Such binding characteristics are not, for example, produced in yeast, insect cells or bacteria. This is because of a very complex relationship between the TSHR's structure and the TSH/TRAb binding sites. The TSHR's post-translational processing and folding of the "mature" protein is best achieved in the mammalian cell environment (see Sanders).

Purification of large amounts of recombinant TSHR from mammalian cells with its TSH and TRAb binding activities intact has not been reported. One of the major problems is loss of TSH/TRAb binding activity following binding with mouse monoclonal antibodies interacting with the extracellular part of the TSHR (Sanders). Also, to date, development of new and convenient strategies for routine measurement of TRAb has not been successful.

EXAMPLES

Preferred features of the present invention are illustrated by the following non-limiting detailed worked examples.

1. Cloning of porcine TSHR cDNA

RNA was extracted from porcine thyroid tissue using the guanidinium thiocyanate-phenol chloroform method (Chomczynski and Sacchi, Anal. Biochem. Vol 162. 1987, pages 156 to 159). mRNA was purified from total RNA using a Dynal bead mRNA purification kit (Dynal, Wirral L62 SAZ UK). This mRNA was used to make a cDNA library using ZapExpress cDNA Gigapack Cloning Kit III (Strategene Ltd., Cambridge CB4 4DF UK). Four degenerate oligonucleotides were made to the known TSHR sequences (mouse, rat, human, dog and bovine) and two fragments of porcine TSHR amplified using PCR. These were sequenced to verify their homology with TSHR cDNA and used to screen the cDNA library for full-length porcine TSHR clones. Three full-length clones were obtained and fully sequenced.

2. Expression of porcine recombinant TSHR protein in CHO cells

An ATG start codon in the 5' untranslated region (5'UTR) of the full-length pTSHR cDNA was removed by PCR and the cDNA cloned into pcDNA 3.1 (+) (Invitrogen BV, 9351 NV Leek, The Netherlands). DNA encoding the full-length TSHR was transfected into CHO cells (CHO-KI from ECACC, Porton Down SP4 OJG UK) by electroporation. Clones expressing TSHR were detected using ^{125}I-TSH binding directly to cells growing on 24-well plates. The clones showing highest TSH binding were expanded and recloned twice by limiting dilution. One stable cell line expressing about 4 x 10^5 TSHR per cell was chosen for expansion and production of recombinant TSHR.

3. Preparation of detergent solubilized recombinant porcine TSHR

CHO cells expressing TSHR were grown to confluence, detached from roller bottles and the cell pellet washed with ice-cold 50 mM NaCl, 10 mM Tris-HCl, pH 7.5 containing 1 mM phenylmethylsulfonylfluoride (PMSF), then homogenized in the same buffer. The cell membranes after centrifugation at 12000g for 30 min at 4°C were solubilized in the same

buffer (4 ml of buffer for approximately 4 x 10^8 cells) as used for homogenization except for addition of 1% Triton X-100. The solubilized receptor preparations were centrifuged at 90,000g for two hours at 4°C and the supernatant stored at -70°C.

4. Expression of the C-terminal end of the porcine TSHR protein

The expression in *Escherichia coli* as a fusion protein with glutathione S-transferase (GST) was carried out using standard protocols (as described in Journal of Molecular Endocrinology (1998) Vol 20, pages 233-244 - Oda). The 3' end of cDNA (1809 to 2295 bp) coding for the last 160 amino acids was cloned in frame with the GST fusion protein in pGEX2T vector (Pharmacia Biotech, St. Albans AL1 3AW UK). An overnight culture of *E. coli* (strain UT580) transformed with pGEX-2T/TSHR plasmids was diluted 1/5 into 2 x YTG medium (16 g Tryptone, 10g yeast extract, 5 g NaCl, 20 g glucose per liter, pH 7.0), incubated for 3 hours at 30°C. Thereafter, isopropyl-3-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM in order to induce protein expression, followed by incubation for a further three hours. The bacterial pellets were resuspended in PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per litre, pH 7.4) containing 1% Triton X-100 and sonicated three times for one minute on ice. The inclusion bodies were pelleted, washed in 4M urea, solubilized in 8M urea and separated on 9% polyacrylamide gels (SDS-polyacrylamide electrophoresis, SDS-PAGE) under reducing conditions. The TSHR/GST fusion proteins (mol. wt. 44 kDa) were electroeluted from polyacrylamide gel slices in 0.1M $NaHCO_3$ and 0.1% SDS pH 7.8, dialyzed against 50 mM Tris-HCl pH 8.0 and stored in aliquots at -70°C.

5. Preparation and purification of monoclonal antibodies to the porcine TSHR

Electroeluted TSHR/GST protein was used to immunize BALB C mice (50 μg per mouse per injection) until the titer of antibody to the TSHR was high. The TSHR antibody level in mouse sera was tested using immunoprecipitation assay based on ^{35}S-labelled TSHR produced in an in vitro transcription/translation system (see the method described in Journal of Clinical Endocrinology and Metabolism, Vol 82 (1997) No. 4, pages 1288-1292 - Prentice).

Mouse spleen cells were fused with mouse myeloma cell line (X63-Ag8.653 from ECACC) and cloned to produce stable hybridomas secreting the TSHR antibody using standard

techniques (Oda). The antibody 4E31 was found to precipitate ^{35}S-TSHR in the immunoprecipitation assay and to react well with the TSHR in Western blotting analysis. 4E31 was purified from hybridoma culture supernatants by chromatography on a Prosep-A (Bioprocessing, Consett DH8 6TJ UK) column. Fab_2 fragments were obtained following digestion with pepsin (pepsin concentration of 1 mg/ml in 70 mM sodium acetate, 50 mM NaCl pH 4.0, IgG to enzyme ratio of 5:1) and chromatography on Prosep A to adsorb intact IgG. 4E31 Fab_2 preparations of about 1 mg/ml were stored at - 70°C in aliquots.

6. TSH receptor binding characteristics of the 4E31 antibody

Solubilized recombinant porcine TSH receptor was incubated with ^{125}I-labelled TSH to form a ^{125}I-labelled TSH-TSH receptor complex. The 4E31 IgG (or control IgG) was immobilized by linking it to magnetic latex beads and these beads (100 μl; 1 μg of 4E31) were incubated with the preformed ^{125}I-labelled TSH-TSH receptor complex (100 μ1). After 1 hr at 37°C, the beads were separated on a magnet, washed and counted for ^{125}I. A sample of the ^{125}I-labelled TSH-TSH receptor complex was precipitated with PEG to determine the amount of free labelled TSH present. Table 1 shows the results obtained:

Table 1

Sample	cpm Bound to Beads
4E31 IgG beads plus:-	
(a) labelled TSH-TSH receptor complex	16,374
(b) labelled TSH only	2,118
Control IgG beads plus labelled TSH-TSH receptor complex	2,433
Labelled TSH-TSH receptor complex precipitated by PEG	16,499
Total cpm in 100 μl of labelled complex	37,073

(Control IgG used was a mouse monoclonal antibody to glutamic acid decarboxylase)

These studies indicated clearly that 4E31 could bind to the TSH receptor at the same time as TSH. Further, 4E31 coupled to a solid support could be used to separate TSH bound to the TSH receptor from free TSH with results similar to those obtained with PEG.

Table 2 shows that in the presence of individual healthy human donor sera, about 55 % of the labelled TSH added bound to tubes coated with 4E31 and recombinant porcine TSH receptor (according to the first embodiment of the invention, scheme ii above). Similar binding was observed in the case of sera from patients with Hashimoto's thyroiditis and patients with systemic lupus erythematosus (SLE). However with sera from the 5 patients with Graves' disease, there was markedly less labelled TSH binding (about 35 %). All 5 sera contained readily detectable amounts of TSH receptor autoantibody as judged by inhibition of TSH binding to native porcine TSH receptor and separation of receptor bound and free TSH with PEG.

Table 2 Binding of labelled TSH to plastic tubes coated with 4E31 $(Fab)_2$ followed by recombinant porcine TSH receptor - effect of different sera.

		inhibition of TSH binding (%)	
Sample	**cpm bound**	**coated tube method (recombinant receptor)**	**PEG method (native receptor)**
Individual normal sera:-			
1	15,171		
2	15,209		
3	15,480		
4	14,768		
5	15,496		
Individual Graves' sera:-			
1	10,132	34	27
2	9,575	37	31
3	12,495	18	21
4	11,478	25	11
5	9,163	40	48
Individual Hashimoto sera:-			
1	14,659		
2	15,215		
3	14,603		
4	15,026		
5	15,370		
Individual SLE sera:-			
1	15,711		
2	15,261		
3	15,091		
4	15,386		
5	15,403		
Total cpm	27,420		

Table 3 shows similar results with tubes coated with native (i.e. non-recombinant) porcine TSH receptor via 4E31. Again, the results obtained with the coated tube assay are similar to those obtained with the PEG separation method.

Table 3 Binding of labelled TSH to plastic tubes coated with 4E31 $(Fab)_2$ followed by native detergent solubilized porcine TSH receptor.

	inhibition of TSH binding (%) with:-	
Sample	native porcine TSH receptor bound to plastic tubes (via 4E31 $(Fab)_2$)	native porcine TSH receptor PEG precipitation assay
Individual normal sera:-		
a	1.5	-0.62
b	2.2	3.2
c	-5.0	3.9
d	1.8	3.2
Graves sera:-		
6	74	71
7	75	72
8	81	76
9	70	73
10	77	88
11	90	83
12	88	72
13	80	76
14	57	70
15	90	86
16	55	72
17	81	68

The inhibiting effect on ^{125}I-TSH binding shown in tables 2 and 3 was dependent on the concentration of TRAb in the serum. As shown in Table 4, increasing amounts of TRAb standard preparation (thyroid stimulating antibody 1st International Standard 90/672) had a dose dependent effect on TSH binding inhibition.

Table 4

TRAb standard dilution (mu/ml)	native porcine TSH receptor (PEG separation method) - inhibition of TSH binding (%)	4E31/recombinant porcine TSH receptor coated tubes - inhibiton of TSH binding (%)
0	0	0
1.25	5.1	5.1
2.5	9.9	12.0
5	16.2	17.0
10	28.4	31.7
20	44.5	51.6
40	69.1	74.8

Direct precipitation of ^{125}I-4E31 Fab_2-TSH complexes

Another example of the invention (according to the second embodiment of the invention, scheme (i) above) is an assay in which 4E31 $(Fab)_2$ is labelled with ^{125}I and reacted with the TSH receptor. This complex is then incubated with TSH receptor autoantibodies in patients' sera and the resulting termolecular complex precipitated by addition of solid phase protein A. An example is shown in Table 5.

Table 5 Reaction between recombinant porcine TSH receptor labelled with ^{125}I-4E31 $(Fab)_2$ and TSH receptor autoantibodies.

Sample	cpm bound
Labelled receptor plus:-	
Individual normal serum:-	
e	1,994
f	2,041
90/672 reference material (mU/ml):	
1.25	2,715
2.5	2,958
5	3,358
10	4,202
20	5,868
40	8,949
Graves' serum:-	
18	12,326
19	13,225
Total cpm	48,428

Graves' sera 18 and 19 gave TSH binding inhibition values of 31% and 73% respectively in the PEG precipitation method using native porcine TSH receptor.

4E31 could be labelled with biotin, bound to streptavidin coated tubes and then reacted with porcine TSH receptor - see the first embodiment of the invention, Scheme (iii) b. Receptor immobilized in this way readily bound ^{125}I-labelled TSH and this binding was inhibited by TSH receptor autoantibodies in patient sera. An example is shown in Table 6.

Table 6 Labelled TSH binding to TSH receptor - bound to streptavidin coated tubes via biotinylated 4E31 $(Fab)_2$ and effect of TRAb.

Serum Sample	**^{125}I-labelled TSH bound (cpm)**
Individual healthy normal sera:-	
g	12,225
h	12,164
TRAb positive Graves' sera:-	
20	2,971
21	7,400
Total cpm	27,339

BRIEF DESCRIPTION OF THE DRAWINGS

Features of the present invention and results obtained will now be described and illustrated with reference to the drawings, in which:

Figure 1 illustrates the correlation between results using a method of monitoring autoantibodies to thyroid stimulating hormone receptor using TRAb coated tubes and a method using PEG precipitation assays, both methods using ^{125}I-labelled bovine TSH. Furthermore, results in different patient groups with the method using TRAb coated tubes are shown.

Figure 2 illustrates results of an ELISA method using TSH receptor coated plates; 2a shows the effect of TRAb standard 90/672 on porcine TSH binding to receptor-coated plates; 2b shows a comparison of an ELISA method and a PEG precipitation assay; and results in different patient groups with a TRAb ELISA method using bovine TSH are shown in 2c.

Figure 3 illustrates the correlation between a conventionally used TRAb assay method and a direct precipitation assay method according to the invention. Furthermore, results in different patient groups with a direct TRAb assay are shown.

What is Claimed is:

1. Method of monitoring autoantibodies to thyroid stimulating hormone (TSH) receptor in a sample of body fluid, comprising, in the following order, the steps of:

(a) providing a TSH receptor selected from the group consisting of:

(i) porcine TSH receptor or a fragment thereof immobilized to a solid phase; and

(ii) TSH receptor which is complexed to a labelled antibody;

(b) incubating said TSH receptor with a sample of body fluid;

(c) reacting said incubated sample of body fluid containing said TSH receptor with at least one binding agent which is capable of binding to said TSH receptor in competitive reaction with TSH receptor autoantibodies (TRAb), or when said TSH receptor is (ii), reacting said incubated sample of body fluid, during or after step (b), with at least one binding agent which can bind to TRAb in such a way as not substantially to interfere with binding of said TRAb to said TSH receptor; and

(d) qualitatively or quantitatively detecting bound TRAb in said reacted incubated sample of body fluid.

2. Method according to claim 1, wherein said sample of body fluid comprises blood, plasma or serum.

3. Method according to claim 1, wherein said solid phase comprises a plastics material, a magnetic material or a non-magnetic material.

4. Method according to claim 1, wherein said labelled antibody comprises an antibody to TSH receptor.

5. Method according to claim 1, wherein said agent capable of binding to said TSH receptor is selected from the group consisting of TRAb, mouse monoclonal antibodies, human monoclonal antibodies, peptides and recombinant antibodies.

6. Method according to claim 1, wherein said agent capable of binding to said TSH receptor is immobilized to a solid phase.

7. Method according to claim 1, wherein said agent capable of binding to said TSH receptor is labelled isotopically.

8. Method according to claim 7, wherein said isotopic label comprises ^{125}I.

9. Method according to claim 1, wherein said agent is labelled non-isotopically by means of an enzyme, a dye, or a fluorescent or chemiluminescent material.

10. Method according to claim 1, wherein said porcine TSH receptor is immobilized, either directly or indirectly, to said solid phase, via an antibody to the TSH receptor.

11. Method according to claim 1, wherein said labelled antibody is indirectly labelled with an organic compound.

12. Method according to claim 11, wherein said organic compound comprises biotin.

13. Method according to claim 11, wherein said organic compound is complexed to a protein with an affinity for said compound.

14. Method according to claim 13, wherein said protein is selected from the group consisting of avidin and streptavidin.

15. Method according to claim 1, wherein said porcine TSH receptor is derived from porcine thyroid tissue.

16. Method according to claim 1, wherein said porcine TSH receptor comprises recombinant material with at least one epitope for TRAb.

17. Method according to claim 1, wherein said TSH receptor comprises recombinant or synthetic material with at least one epitope for TRAb.

18. Method according to claim 1, wherein step (d) comprises detecting of labelled or unlabelled TSH bound to TSH receptor and unbound labelled or unlabelled TSH.

19. Method according to claim 18, wherein said labelled or unlabelled TSH comprises bovine or porcine TSH.

20. Method according to claim 1, wherein step (d) comprises detecting of labelled or unlabelled TSH agonist bound to TSH receptor and unbound labelled or unlabelled TSH agonist.

21. Method according to claim 20, wherein said TSH agonist is a monoclonal antibody reactive with said TSH receptor, or a fragment of said monoclonal antibody.

22. Method according to claim 21, in which said monoclonal antibody is a human monoclonal antibody.

23. Method according to claim 21, wherein said monoclonal antibody is a recombinant antibody or recombinant antibody fragment.

24. Method according to claim 20, wherein said TSH agonist is a peptide.

25. Method according to claim 24, wherein said peptide is derived from TSH.

26. Method according to claim 1, wherein step (d) comprises detecting of labelled or unlabelled TSH antagonist bound to TSH receptor and unbound labelled or unlabelled TSH antagonist.

27. Method according to claim 26, wherein said TSH antagonist is a monoclonal antibody reactive with said TSH receptor, or a fragment of said monoclonal antibody.

28. Method according to claim 27, in which said monoclonal antibody is a human monoclonal antibody.

29. Method according to claim 27, wherein said monoclonal antibody is a recombinant antibody or recombinant antibody fragment.

30. Method according to claim 26, wherein said TSH antagonist is a peptide.

31. Method according to claim 30, wherein said peptide is derived from TSH.

32. A kit for monitoring autoantibodies to thyroid stimulating hormone (TSH) receptor in a sample of body fluid, comprising:

(a) providing a TSH receptor selected from the group consisting of:

(i) porcine TSH receptor or a fragment thereof immobilized to a solid phase; and

(ii) TSH receptor which is complexed to a labelled antibody;

(b) at least one binding agent which is capable of binding to said TSH receptor in competitive reaction with TSH receptor autoantibodies (TRAb), or when said TSH receptor is (ii) at least one binding agent which can bind to TRAb in such a way as not substantially to interfere with binding of said TRAb to said TSH receptor;

(c) means for incubating said TSH receptor of (a) with a sample of body fluid;

(d) means for reacting said incubated sample of body fluid of (c) with said at least one binding agent of (b); and

(e) means for qualitatively or quantitatively detecting bound TRAb in said reacted incubated sample of body fluid.

33. A kit according to claim 32, wherein said sample of body fluid comprises blood, plasma or serum.

34. A kit according to claim 32, wherein said solid phase comprises a plastics material, a magnetic material or a non-magnetic material.

35. A kit according to claim 32, wherein said labelled antibody comprises an antibody to TSH receptor.

36. A kit according to claim 32, wherein said agent capable of binding to said TSH receptor is selected from the group consisting of TRAb, mouse monoclonal antibodies, human monoclonal antibodies, peptides and recombinant antibodies.

37. A kit according to claim 32, wherein said agent capable of binding to said TSH receptor is immobilized to a solid phase.

38. A kit according to claim 32, wherein said agent capable of binding to said TSH receptor is labelled isotopically.

39. A kit according to claim 38, wherein said isotopic label comprises ^{125}I.

40. A kit according to claim 32, wherein said agent is labelled non-isotopically by means of an enzyme, a dye, or a fluorescent or chemiluminescent material.

41. A kit according to claim 32, wherein said porcine TSH receptor is immobilized, either directly or indirectly, to said solid phase, via an antibody to the TSH receptor.

42. A kit according to claim 32, wherein said labelled antibody is indirectly labelled with an organic compound.

43. A kit according to claim 42, wherein said organic compound comprises biotin.

44. A kit according to claim 42, wherein said organic compound is complexed to a protein with an affinity for said compound.

45. A kit according to claim 44, wherein said protein is selected from the group consisting of avidin and streptavidin.

46. A kit according to claim 32, wherein said porcine TSH receptor is derived from porcine thyroid tissue.

47. A kit according to claim 32, wherein said porcine TSH receptor comprises recombinant material with at least one epitope for TRAb.

48. A kit according to claim 32, wherein said TSH receptor comprises recombinant or synthetic material with at least one epitope for TRAb.

49. A kit according to claim 32, wherein step (d) comprises detecting of labelled or unlabelled TSH bound to TSH receptor and unbound labelled or unlabelled TSH.

50. A kit according to claim 49, wherein said labelled or unlabelled TSH comprises bovine or porcine TSH.

51. A kit according to claim 32, wherein step (d) comprises detecting of labelled or unlabelled TSH agonist bound to TSH receptor and unbound labelled or unlabelled TSH agonist.

52. A kit according to claim 51, wherein said TSH agonist is a monoclonal antibody reactive with said TSH receptor, or a fragment of said monoclonal antibody.

53. A kit according to claim 52, in which said monoclonal antibody is a human monoclonal antibody.

54. A kit according to claim 52, wherein said monoclonal antibody is a recombinant antibody or recombinant antibody fragment.

55. A kit according to claim 51, wherein said TSH agonist is a peptide.

56. A kit according to claim 55, wherein said peptide is derived from TSH.

57. A kit according to claim 32, wherein step (d) comprises detecting of labelled or unlabelled TSH antagonist bound to TSH receptor and unbound labelled or unlabelled TSH antagonist.

58. A kit according to claim 57, wherein said TSH antagonist is a monoclonal antibody reactive with said TSH receptor, or a fragment of said monoclonal antibody.

59. A kit according to claim 58, in which said monoclonal antibody is a human monoclonal antibody.

60. A kit according to claim 58, wherein said monoclonal antibody is a recombinant antibody or recombinant antibody fragment.

61. A kit according to claim 57, wherein said TSH antagonist is a peptide.

62. A kit according to claim 61, wherein said peptide is derived from TSH.

ABSTRACT OF THE DISCLOSURE

Assays for TSH Receptor Autoantibodies

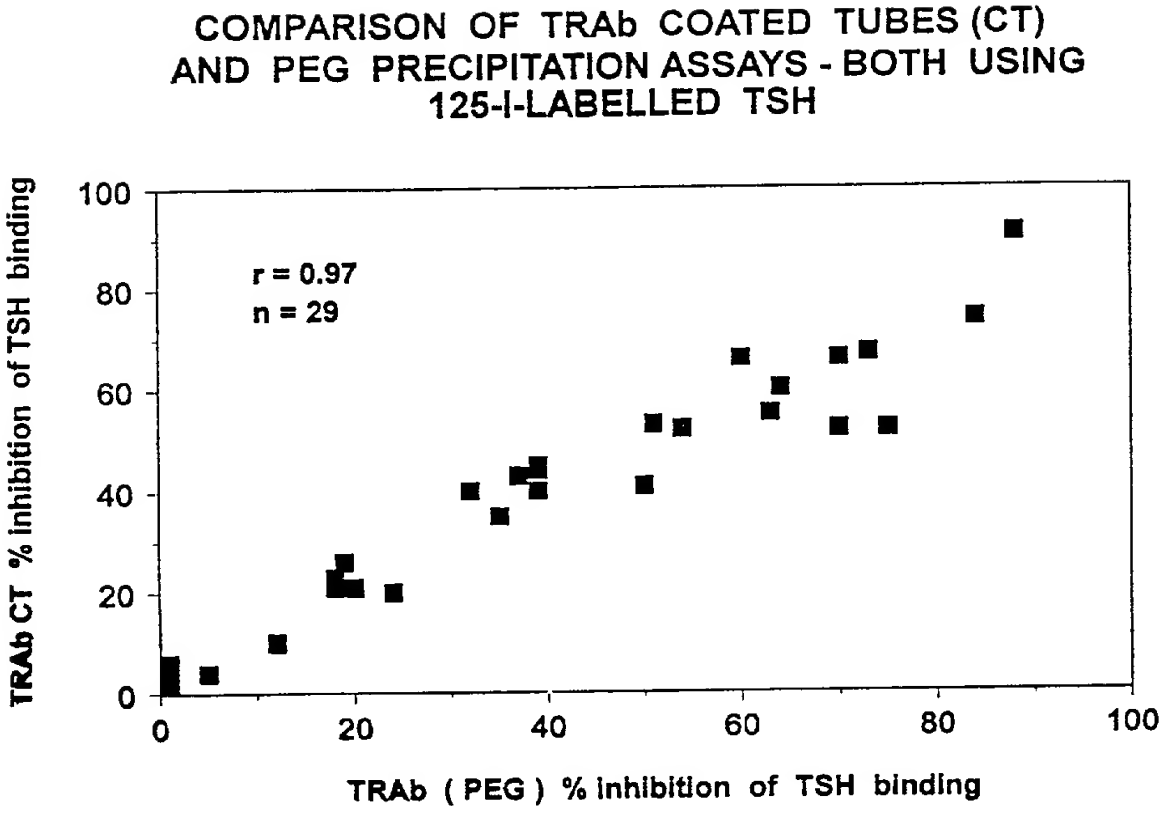
A method and kit for monitoring autoantibodies to thyroid stimulating hormone (TSH) receptor in a sample of body fluid, which employs the steps of:

(i) incubating TSH receptor with a sample of body fluid;

(ii) reacting the incubated sample of body fluid with at least one binding agent which is capable of binding to the TSH receptor in competitive reaction with TSH receptor autoantibodies (TRAb), or in a case where TSH receptor is complexed to labelled antibody, reacting the sample of body fluid with at least one binding agent which can bind to TRAb in such a way as not substantially to interfere with binding of the TRAb to the TSH receptor; and

(iii) detecting bound TRAb in the reacted incubated sample of body fluid.

Figure 1


COMPARISON OF TRAb COATED TUBES (CT) AND PEG PRECIPITATION ASSAYS - BOTH USING 125-I-LABELLED TSH
TRAb CT % inhibition of TSH binding
TRAb (PEG) % inhibition of TSH binding
r = 0.97
n = 29
0
20
40
60
80
100

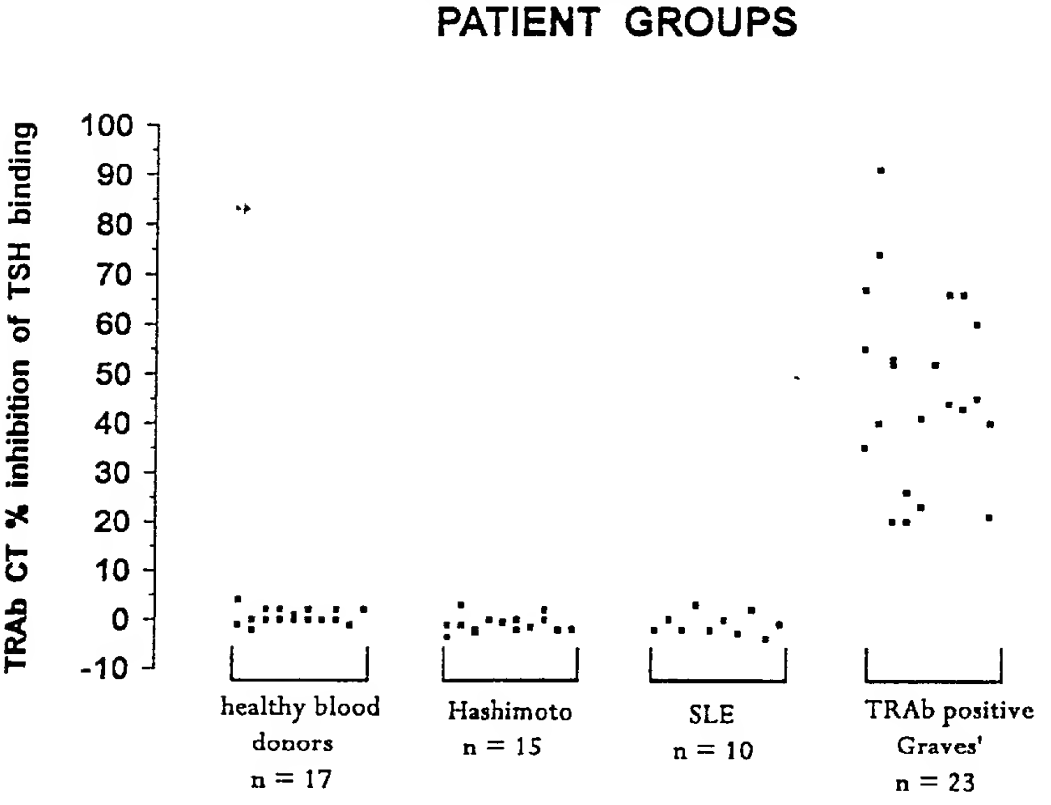
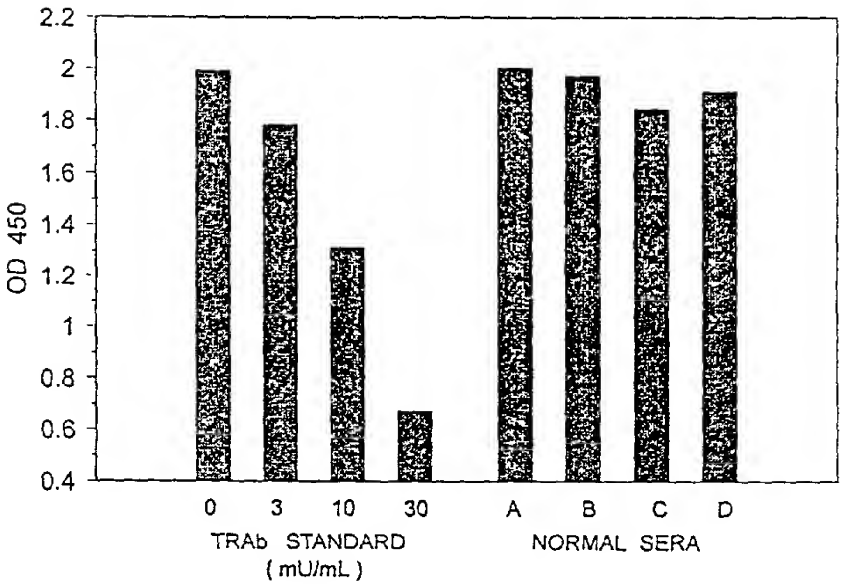
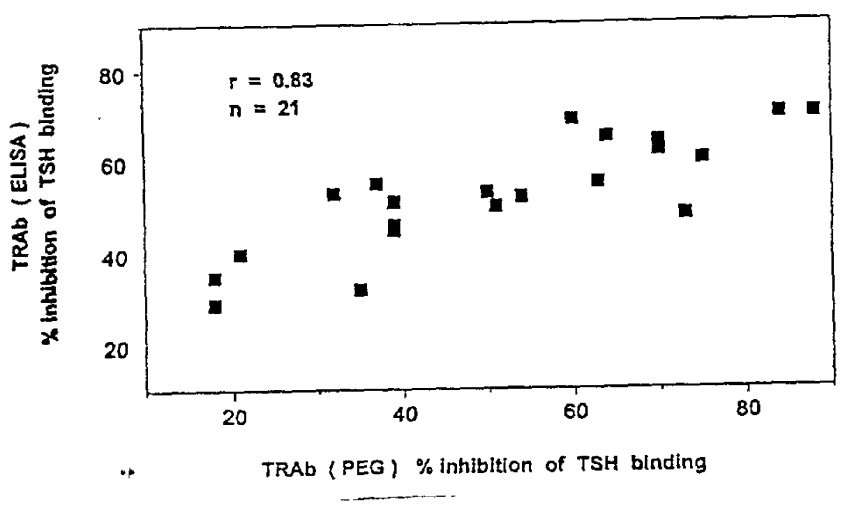

TRAb COATED TUBE ASSAY - RESULTS IN DIFFERENT PATIENT GROUPS
TRAb CT % inhibition of TSH binding
100
90
80
70
60
50
40
30
20
10
0
-10
healthy blood donors n = 17
Hashimoto n = 15
SLE n = 10
TRAb positive Graves' n = 23

Figure 2

TRAb ELISA USING TSH RECEPTOR COATED PLATES
INHIBITION OF PORCINE TSH-PEROXIDASE BINDING
BY TRAb STANDARD 90/672



COMPARISON OF ELISA AND PEG PRECIPITATION ASSAYS
FOR TRAb USING 21 GRAVES SERA



TRAb ELISA RESULTS IN DIFFERENT PATIENT GROUPS

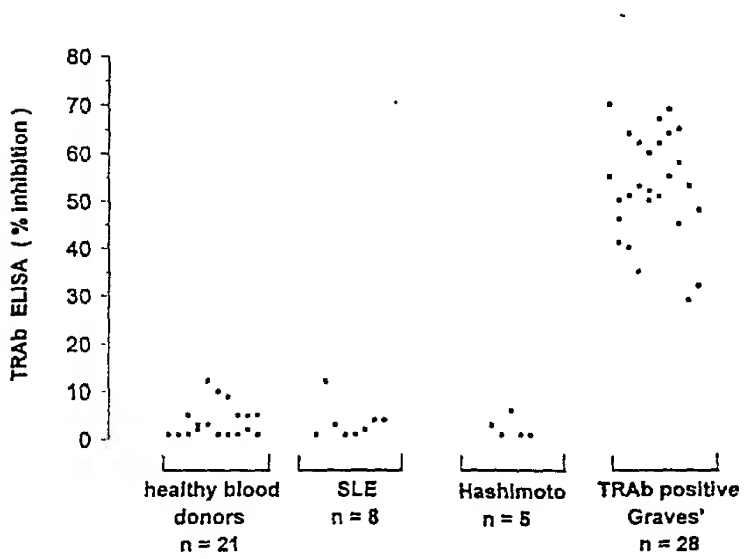
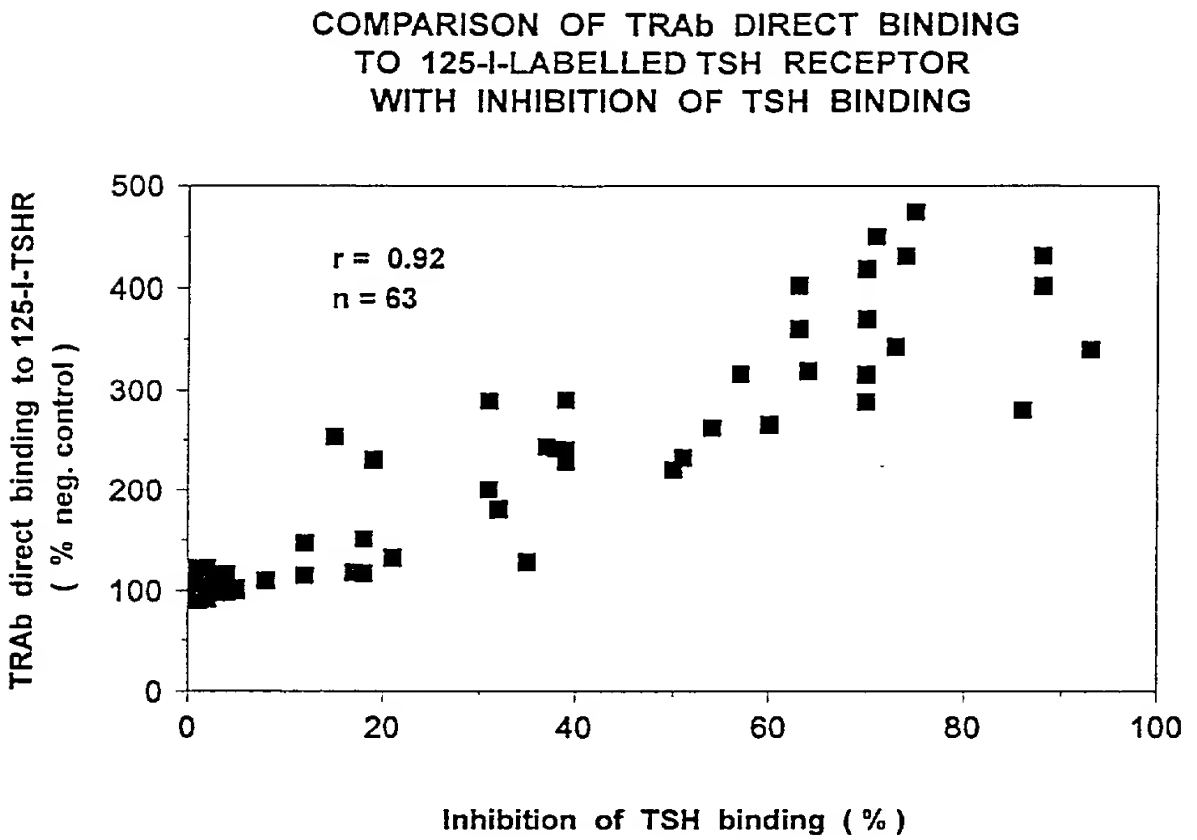
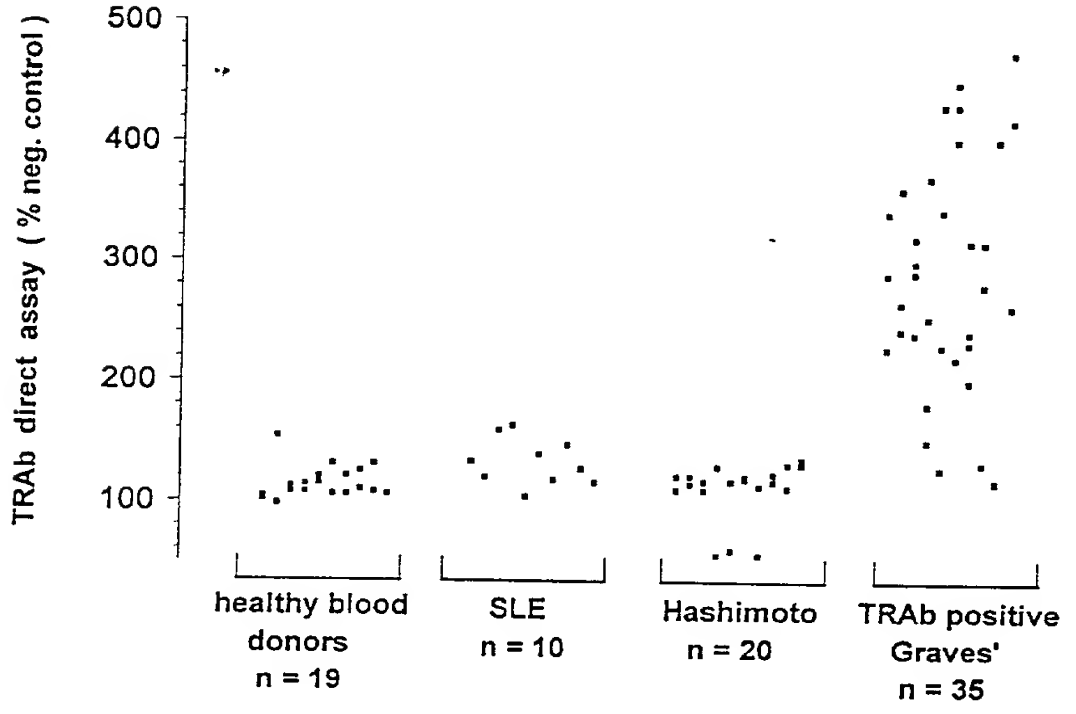


Figure 3


COMPARISON OF TRAb DIRECT BINDING
TO 125-I-LABELLED TSH RECEPTOR
WITH INHIBITION OF TSH BINDING
TRAb direct binding to 125-I-TSHR
(% neg. control)
r = 0.92
n = 63
500
400
300
200
100
0
0
20
40
60
80
100
Inhibition of TSH binding (%)


DIRECT TRAb ASSAY - RESULTS IN DIFFERENT
PATIENT GROUPS
TRAb direct assay (% neg. control)
500
400
300
200
100
healthy blood
donors
n = 19
SLE
n = 10
Hashimoto
n = 20
TRAb positive
Graves'
n = 35

DECLARATION AND POWER OF ATTORNEY

FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Assays for TSH Receptor Autoantibodies

the specification of which is filed herewith.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations. §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign applications for patent listed below, and I have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

United Kingdom	9812146.0	6th June 1998
United Kingdom	9909661.2	28th April 1999

And I hereby appoint:

Kenneth I. Kohn of Kohn & Associates, 30500 Northwestern Hwy., Suite 410, Farmington Hills, Michigan 48334, as my attorney, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all business in the Patent and Trademark office connected therewith. I hereby revoke any and all previous declarations and powers of attorney signed in connection with the subject application

Please address all communications, and direct all telephone calls regarding this application, to:

Kenneth I. Kohn
Kohn & Associates
30500 Northwestern Hwy
Suite 410
Farmington Hills
MI 48334

Tel: (810) 539-5050

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of First Inventor

SMITH, Bernard Rees

Residence	)	United Kingdom
Post Office	)	Richmond House
Address	)	Druidstone Road
	)	St. Mellons
	)	Cardiff CF3 9XE
	)	United Kingdom
Citizenship	)	British

Dated this 25th day of January 2000 ~~1999~~

Bernard Rees Smith

Full name of Second inventor

SANDERS, Jane

Residence	)	United Kingdom
Post Office	)	21 Lowfield Drive
Address	)	Thornhill
	)	Cardiff
	)	CF4 9HT
	)	United Kingdom
Citizenship	)	British

Dated this 27th day of January 2000 ~~1999~~

Jane Sanders

Jane Sanders

Full name of Third Inventor

FURMANIAK, Jadwiga

Residence	)	United Kingdom
Post Office	)	35 Havenwood Drive
Address	)	Thornhill
	)	Cardiff CF4 9HZ
	)	United Kingdom
Citizenship	)	British

Dated this 27th day of January 2000 ~~1999~~

Jadwiga Furmaniak

Applicant or Patentee . Jane Sanders et al.
Serial or Patent No.
Filed or Issued .
For . Assays for TSH Receptor Autoantibodies

Attorney's Docket No 0769.00136

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9 (f) and 1.27 (c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

[ ] the owner of the small business concern identified below:
[x] an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN RSR Limited,
ADDRESS Avenue Park, Pentwyn, Cardiff CF2 7HE.
United Kingdom.

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9 (d), for purposes of paying reduced fees under section 41 (a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed in a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, described in

[x] the specification entitled "Assays for TSH Receptor Autoantibodies" to be filed as a United States Patent Application in the name of Bernard Rees Smith *et al*

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by an person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9 (d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9 (d) or a nonprofit organization under 37 CFR 1.9 (e).

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Bernard Rees Smith
TITLE OF PERSON OTHER THAN OWNER Director
ADDRESS OF PERSON SIGNING Richmond House
Druidstone Road
St. Mellons
Cardiff CF3 9XE
United Kingdom

SIGNATURE _____ DATE Janu 25th 2000

Applicant : Jane Sanders et al.
Serial or Patent No. :
Filed or Issued :
Title : Assays for TSH Receptor Autoantibodies

Attorney's Docket No 0769.00136

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9 (f) and 1.27 (b)) - INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9 (c) for purposes of paying reduced fees under section 41 (a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled Diagnosis of Autoimmune Adrenal Disease described in

[x] the specification entitled "Assays for TSH Receptor Autoantibodies" to be filed as a United States Patent Application in the name of Bernard Rees Smith *et al*

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9 (c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9 (d) or a nonprofit organization under 37 CFR 1.9 (e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

[ ] no such person, concern, or organization
[x] persons, concerns or organizations listed below*

*NOTE: Separate verified statements are required from each named person concern or organization having rights to the invention averring to their status as small entities (37 CFR 1.27)

FULL NAME RSR Limited
ADDRESS Avenue Park, Pentwyn, Cardiff CF2 7HE, United Kingdom

[ ] Individual [x] Small Business Concern [ ] Non-Profit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Bernard Rees Smith
Name of Inventor

Signature of Inventor

January 25th 2000
Date

Jane Sanders
Name of Inventor

Jane Sanders
Signature of Inventor

27th January 2000
Date

Jadwiga Furmaniak
Name of Inventor

Signature of Inventor

27th January 2000
Date